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<b>(21) International Application Number:</b> PCT/SE96/01558 <b>(22) International Filing Date:</b> 28 November 1996 (28.11.96) <b>(30) Priority Data:</b> 9504272-7                      29 November 1995 (29.11.95)      SE 9601506-0                      19 April 1996 (19.04.96)                      SE <b>(71) Applicant (for all designated States except US):</b> AMYLOGENE HB [SE/SE]; c/o Svalöf Weibull AB, S-268 81 Svalöv (SE). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> EK, Bo [SE/SE]; Ny- hagen, S-740 30 Björklinge (SE). KHOSNOODI, Jamshid [SE/SE]; Bandstolsvägen 3, 2 tr., S-756 48 Uppsala (SE). LARSSON, Clas-Tomas [SE/SE]; Flogstavägen 55 B II, S- 752 73 Uppsala (SE). LARSSON, Håkan [SE/SE]; Hammar- bygatan 58, S-753 24 Uppsala (SE). RASK, Lars [SE/SE]; Säves väg 14, S-752 63 Uppsala (SE). <b>(74) Agent:</b> AWAPATENT AB; P.O. Box 5117, S-200 71 Malmö (SE).		<b>(81) Designated States:</b> AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> STARCH BRANCHING ENZYME II OF POTATO		
<b>(57) Abstract</b> <p>The present invention relates to an amino acid sequence of second starch branching enzyme (SBE II) of potato and a fragment thereof as well as to the corresponding isolated DNA sequences. Furthermore, the invention relates to vectors comprising such an isolated DNA sequence, to processes for production of transgenic potatoes, and to the use of said potatoes for the production of starch. The starch obtained will show a changed pattern of branching of amylopectin as well as a changed amylose/amylopectin ratio.</p>		

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## STARCH BRANCHING ENZYME II OF POTATO

The present invention relates to a novel starch branching enzyme of potato. More specifically, the present invention relates to an amino acid sequence of a second starch branching enzyme (SBE II) of potato and a fragment thereof as well as their corresponding DNA sequences. Furthermore, the invention relates to vectors comprising such DNA sequences, to processes for production of transgenic potatoes, and to the use of said potatoes for the production of starch.

Starch is a complex mixture of different molecule forms differing in degree of polymerization and branching of the glucose chains. Starch consists of amylose and amylopectin, whereby the amylose consists of an essentially linear  $\alpha$ -1,4-glucan and amylopectin consists of  $\alpha$ -1,4-glucans connected to each other via  $\alpha$ -1,6-linkages and, thus, forming a branched polyglucan. Thus, starch is not a uniform raw material.

Starch is synthesized via at least three enzymatic reactions in which ADP glucose phosphorylase (EC 2.7.7.27), starch synthase (EC 2.4.1.21) and starch branching enzyme (EC 2.4.1.18) are involved. Starch branching enzyme (SBE, also called Q-enzyme) is believed to have two different enzymatic activities. It catalyzes both the hydrolysis of  $\alpha$ -1,4-glucosidic bonds and the formation of  $\alpha$ -1,6-glucosidic bonds during synthesis of the branched component in starch, i.e. amylopectin.

Plant starch is a valuable source of renewable raw material used in, for example, the chemical industry (Visser and Jacobsen, 1993). However, the quality of the starch has to meet the demands of the processing industry wherein uniformity of structure is an important criterion. For industrial application there is a need of plants only containing amylose starch and plants only containing amylopectin starch, respectively.

Processes for altering the amylose/amylopectin ratio in starch have already been proposed. For example, in WO95/04826 there is described DNA sequences encoding debranching enzymes with the ability to reduce or increase the degree of branching of amylopectin in transgenic plants, e.g. potatoes.

In WO92/14827 plasmids are described having DNA sequences that after insertion into the genome of the plants cause changes in the carbohydrate concentration and the carbohydrate composition in regenerated plants. These changes can be obtained from a sequence of a branching enzyme that is located on these plasmids. This branching enzyme is proposed to alter the amylose/amylopectin ratio in starch of the plants, especially in commercially used plants.

WO92/14827 describes the only hitherto known starch branching enzyme in potato and within the art it is not known whether other starch branching enzymes are involved in the synthesis of branched starch of potato.

In Mol Gen Genet (1991) 225:289-296, Visser et al., there is described inhibition of the expression of the gene for granule-bound starch synthase in potato by antisense constructs. Inhibition of the enzyme in potato tuber starch was up to 100% in which case amylose-free starch was provided.

However, the prior known methods for inhibiting amylopectin have not been that successful and, therefore, alternative methods for inhibiting amylopectin are still highly desirable (Müller-Röber and Koßmann, 1994; Martin and Smith, 1995).

The object of the present invention is to enable altering the degree of amylopectin branching and the amylopectin/amylose ratio in potato starch.

According to the present invention this object is achieved by providing a novel isolated DNA sequence encoding a second starch branching enzyme, SBE II, and

fragments thereof, which after insertion into the genome of the plants cause changes in said branching degree and ratio in regenerated plants.

Within the scope of the present invention there is  
5 also included the amino acid sequence of SBE II and fragments thereof.

Also variants of the above DNA sequence resulting from the degeneracy of the genetic code are encompassed.

The novel DNA sequence encoding SBEII, comprising  
10 3074 nucleotides, as well as the corresponding amino acid sequence comprising 878 amino acids, are shown in SEQ ID No. 1. One 1393 nucleotides long fragment of the above DNA sequence, corresponding to nucleotides 1007 to 2399 of the DNA sequence in SEQ ID No. 1, as well as the corresponding  
15 amino acid sequence comprising 464 amino acids, are shown in SEQ ID No. 2.

Furthermore, there are provided vectors comprising said isolated DNA-sequences and regulatory elements active in potato. The DNA sequences may be inserted in the sense  
20 or antisense (reversed) orientation in the vectors in relation to a promoter immediately upstream from the DNA sequence.

Also there is provided a process for the production of transgenic potatoes with a reduced degree of branching  
25 of amylopectin starch, comprising the following steps:  
a) transfer and incorporation of a vector according to the invention into the genome of a potato cell, and  
b) regeneration of intact, whole plants from the transformed cells.

30 Finally, the invention provides the use of said transgenic potatoes for the production of starch.

The invention will be described in more detail below in association with an experimental part and the accompanying drawings, in which

35 Fig. 1 shows SDS polyacrylamide electrophoresis of proteins extracted from starch of normal potato (lane A)

and transgenic potato (lane B). Excised protein bands are marked with arrows. Lane M: Molecular weight marker proteins (kDa).

Fig. 2 shows 4 peptide sequences derived from  
5 digested proteins from potato tuber starch.

#### EXPERIMENTAL PART

##### *Isolation of starch from potato tubers*

Potato plants (*Solanum tuberosum*) were grown in the  
10 field. Peeled tubers from either cv. Early Puritan or from a transgenic potato line essentially lacking granule-bound starch synthase I (Svalöf Weibull AB, international application number PCT/SE91/00892), were homogenized at 4°C in a fruit juicer. To the "juice fraction", which  
15 contained a large fraction of the starch, was immediately added Tris-HCl, pH 7.5, to 50 mM, Na-dithionite to 30 mM and ethylenedinitrilotetraacetic acid (EDTA) to 10 mM. The starch granules were allowed to sediment for 30 min and washed 4x with 10 bed volumes of washing buffer (50 mM  
20 Tris-HCl, pH 7.5, 10 mM EDTA). The starch, which was left on the bench at +4°C for 30 min to sediment between every wash, was finally washed with 3 x 3 bed volumes of acetone, air dried over night, and stored at -20°C.

##### *Extraction of proteins from tuber starch*

25 Stored starch (20 g) was continuously mixed with 200 ml extraction buffer (50 mM Tris-HCl, pH 7.5, 2% (w/v) sodium dodecyl sulfate (SDS), 5 mM EDTA) by aspiration with a pipette at 85°C until the starch was gelatinized. The samples were then frozen at -70°C for 1 hour. After  
30 thawing at 50°C, the samples were centrifuged for 20 min at 12,000xg at 10°C. The supernatants were collected and re-centrifuged at 3,000xg for 15 min. The final supernatants were filtered through 0.45 µ filters and 2.25 volumes of ice-cold acetone were added. After 30 min  
35 incubation at 4°C, the protein precipitates were collected by centrifugation (3,000xg for 30 min at 4°C), and

dissolved in 50 mM Tris-HCl, pH 7.5. An aliquot of each preparation was analyzed by SDS poly-acrylamide gel electrophoresis according to Laemmli (1970) (Fig. 1). The proteins in the remaining portions of the preparations were concentrated by precipitation with trichloroacetic acid (10%) and the proteins were separated on an 8% SDS polyacrylamide gel Laemmli, (1970). The proteins in the gel were stained with Coomassie Brilliant Blue R-250 (0.2% in 20% methanol, 0.5% acetic acid, 79.5% H<sub>2</sub>O).

10 *In gel digestion and sequencing of peptides*

The stained bands marked with arrows in Fig. 1 corresponding to an apparent molecular weight of about 100 kDa were excised and washed twice with 0.2M NH<sub>4</sub>HCO<sub>3</sub> in 50% acetonitrile under continuous stirring at 35°C for 20 min. After each washing, the liquid was removed and the gel pieces were allowed to dry by evaporation in a fume hood. The completely dried gel pieces were then separately placed on parafilm and 2 µl of 0.2M NH<sub>4</sub>CO<sub>3</sub>, 0.02% Tween-20 were added. Modified trypsin (Promega, Madison, WI, USA) (0.25 µg in 2 µl) was sucked into the gel pieces whereafter 0.2M NH<sub>4</sub>CO<sub>3</sub> was added in 5 µl portions until they had resumed their original sizes. The gel slices were further divided into three pieces and transferred to an Eppendorf tube. 0.2M NH<sub>4</sub>CO<sub>3</sub> (200 µl) was added and the proteins contained in the gel pieces were digested over night at 37°C (Rosenfeld et al. 1992). After completed digestion, trifluoroacetic acid was added to 1% and the supernatants removed and saved. The gel pieces were further extracted twice with 60% acetonitrile, 0.1% trifluoroacetic acid (200 µl) under continuous shaking at 37°C for 20 min. The two supernatants from these extractions were combined with the first supernatant. The gel pieces were finally washed with 60% acetonitrile, 0.1% trifluoroacetic acid, 0.02% Tween-20 (200 µl). Also these supernatants were combined with the other supernatants and the volume was reduced to 50 µl by evaporation. The

extracted peptides were separated on a SMART<sup>®</sup> chromatography system (Pharmacia, Uppsala, Sweden) equipped with a  $\mu$ RPC C2/C18 SC2.1/10 column. Peptides were eluted with a gradient of 0 - 60% acetonitrile in water/0.1% trifluoroacetic acid over 60 min with a flow rate of 100  $\mu$ l/min. Peptides were sequenced either on an Applied Biosystems 470A gas phase sequenator with an on line PTH-amino acid analyzer (120A) or on a model 476A according to the instructions of the manufacturer (Applied Biosystems, Foster City, CA, USA).

Four of the peptides sequenced gave easily interpretable sequences (Fig. 2). A data base search revealed that these four peptides displayed similarity to starch branching enzymes and interestingly, the peptides were more related to starch branching enzyme II from other plant species than to starch branching enzyme I from potato.

*Construction of oligonucleotides encoding peptides 1 and 2.*

Degenerated oligonucleotides encoding peptide 1 and peptide 2 were synthesized as forward and reverse primers, respectively:

Oligonucleotide 1: 5'-gtaaaacgacggccagt-TTYGGNGTNTGGGARATHTT-3' (Residues 2 to 8 of peptide 1)

Oligonucleotide 2: 5'-aattaaccctcactaaaggg-CKRTCRAAYTCYTGARNCC-3' (Residues 2 to 8 of peptide 2, reversed strand)

wherein

H is A, C or T, I is inosine; K is G or T; N is A, C, G or T; R is A or G; Y is C or T; bases in lower case were added as tag sequences.



*Purification of mRNA from potato tuber, synthesis of cDNA and PCR amplification of a cDNA fragment corresponding to potato starch branching enzyme II.*

Total RNA from mature potato tubers (*S. tuberosum* cv. Amanda) was isolated as described (Logemann et al. 1987). First strand cDNA was synthesized using 2 µg of total RNA and 60 pmol of oligo-dT<sub>30</sub> as downstream primer. The primer was annealed to the polyA of the mRNA at 60°C for 5 min. The extension of the cDNA was performed according to the technical manual of the manufacturer using the Riboclone<sup>®</sup> cDNA Synthesis System M-MLV (H-) (Promega).

cDNA encoding the novel starch branching enzyme II according to the invention was amplified in a Perkin-Elmer GeneAmp<sup>®</sup> 9600 PCR thermocycler (Perkin-Elmer Cetus Instruments, CT, USA) using the two degenerate primers designed from the peptides 1 and 2 (see above) under the following conditions: 1 mM dNTP, 1 µM of each primer and an aliquot of the cDNA described above in a total reaction volume of 20 µl with 1x AmpliTaq<sup>®</sup> buffer and 0,8 U AmpliTaq<sup>®</sup> (Perkin-Elmer Cetus). The cycling conditions were: 96°C for 1', 80°C while the enzyme was added as a hotstart (approximately 15'), an unintended drop to 25°C, five cycles of 94°C for 20", 45°C for 1', ramp to 72°C for 1' and 72°C for 2', and 30 cycles of 94°C for 5", 45°C for 30", and 72°C for (2'+2" per cycle) and completed with 72°C for 10' prior to chilling to 4°C.

A sample of this reaction (0.1 µl) was reamplified using the cycling conditions: 96°C for 1', 80°C while the enzyme was added as a hotstart (approximately 5'), five cycles of 94°C for 20'', 45°C for 1', and 72°C for 2', and 25 cycles of 94°C for 5'', 45°C for 30'', and 72°C for (2' + 2'' per cycle) and completed with 72°C for 10' prior to chilling to 4°C. After completion of the PCR amplification, the reaction was loaded on a 1.5% Seakem<sup>®</sup> agarose gel (FMC Bioproducts, Rockland, ME, USA). After electrophoresis and staining with ethidium bromide a major

band with an apparent size of 1500 bp was excised and the fragment was eluted by shaking in water (200 µl) for 1 h. This fragment was used as template in sequencing reactions after reamplification using primers corresponding to the tag sequences (in oligonucleotides 1 and 2), purification by agarose gel electrophoresis as above and extraction from the gel using the Qiaex® gel extraction kit according to the manufacturer's instructions (DIAGEN GmbH, Hilden, Germany). The sequencing reactions were done using the DyeDeoxy® Terminator Cycle Sequencing kits (Perkin-Elmer Cetus Instruments) using tag sequences and internal primers. The sequencing reaction were analyzed on an Applied Biosystems 373A DNA sequencer according to the manufacturer's protocols. The sequence was edited and comprised 1393 bp.

To complete the determination of the sequence of starch branching enzyme II, the 5' and 3' ends of the full length cDNA were amplified from the same total RNA as above using rapid amplification of cDNA ends, RACE, methodology with specific primers from the 1393 bp sequence. In the 3' end amplification, an oligo T<sub>20</sub>G primer was used against the poly A tail and in the 5' end, the 5'/3' RACE kit from Boehringer Mannheim (Cat. No. 1734792) was used. The fragments from these amplifications were sequenced in the same way as above using internal and end primers. The sequences from the two ends were aligned together with the 1393 base pairs to give a composite full length cDNA sequence. Primers were designed from this sequence to amplify the whole coding region in one part. Partial sequencing of the amplified coding cDNA confirmed the presence of a cDNA corresponding to the composite sequence. The full length cDNA is 3074 bp and the translated sequence comprises 878 amino acids. The mature protein comprises 830 amino acids.

Comparisons of the consensus sequence with the EMBL and GenBank databases showed 68% identity to potato starch

branching enzyme I and about 80% identity to starch  
branching enzyme II from other plant species. The present  
inventors therefore denote the enzyme encoded by the new  
branching enzyme sequence potato starch branching enzyme  
5 II.

*Transformation of potato plants*

The isolated full length cDNA of potato starch  
branching enzyme II and other functionally active frag-  
ments in the range of 50-3 074 bp are cloned in reverse  
10 orientation behind promoters active in potato tubers. By  
the term "functionally active" is meant fragments that  
will affect the amylose/amylopectin ratio in potato  
starch. The DNA and amino acid sequence of SBE II  
according to the invention as well as one fragment of the  
15 DNA and corresponding amino acid sequence are shown in SEQ  
ID No. 1 and 2, respectively.

The promoters are selected from, for example, the  
patatin promoter, the promoter from the potato granule-  
bound starch synthase I gene or promoters isolated from  
20 potato starch branching enzymes I and II genes.

The constructs are cloned by techniques known in the  
art either in a binary Ti-plasmid vector suitable for  
transformation of potato mediated by *Agrobacterium*  
*tumefaciens*, or in a vector suitable for direct  
25 transformation using ballistic techniques or  
electroporation. It is realized that the sense (see below)  
and antisense constructs must contain all necessary  
regulatory elements.

Transgenic potato plants transcribe the inverse  
30 starch branching enzyme II construct specifically in  
tubers, leading to antisense inhibition of the enzyme. A  
reduction and changed pattern of the branching of amylo-  
pectin as well as a changed amylose/amylopectin ratio  
thereby occur in tuber starch.

35 The antisense construct for potato starch branching  
enzyme II is also used in combination with antisense

constructs for potato starch branching enzyme I, for  
potato granule-bound starch synthase II, for potato  
soluble starch synthases II and III, for potato starch  
disproportionating enzyme (D-enzyme) or for potato starch  
5 debranching enzyme to transform potato to change the  
degree of branching of amylopectin and the  
amylose/amylopectin ratio. This gives new and valuable raw  
material to the starch processing industry.

The full-length cDNA sequence encoding the enzyme is,  
10 in different constructs, cloned in sense orientation  
behind one or more of the promoters mentioned above, and  
the constructs are transferred into suitable transforma-  
tion vectors as described above and used for the  
transformation of potato. Regenerated transformed potato  
15 plants will produce an excess of starch branching enzyme  
II in the tubers leading to an increased degree and  
changed pattern of branching of amylopectin or to  
inhibition of transcription of endogenous starch branching  
enzyme II transcription due to co-suppression, resulting  
20 in a decreased branching of amylopectin.

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SEQ ID No. 1

Sequenced molecule: cDNA

Name: beII gene (branching enzyme II) from *Solanum tuberosum* (potato)

Length of sequence: 3074 bp

AAACCTCCTC CACTCAGTCT TTGTTTCTCT CTCTCTTCAC GCTTCTCTTG GCGCCTTGAA	60
CTCAGCAATT TGACACTCAG TTAGTTACAC TNCCATCACT TATCAGATCT CTATTTTTC	120
TCTTAATTC AACCAAGGAA TGAATAAAAA GATAGATTG TAAAAACCT AAGGAGAGAA	180
GAAGAAAG ATG GTG TAT ACA CTC TCT GGA GTT CGT TTT CCT ACT GTT CCA	230
Met Val Tyr Thr Leu Ser Gly Val Arg Phe Pro Thr Val Pro	
-45 -40 -35	
TCA GTG TAC AAA TCT AAT GGA TTC AGC AGT AAT GGT GAT CGG AGG AAT	278
Ser Val Tyr Lys Ser Asn Gly Phe Ser Ser Asn Gly Asp Arg Arg Asn	
-30 -25 -20	
GCT AAT NTT TCT GTA TTC TTG AAA AAG CAC TCT CTT TCA CGG AAG ATC	326
Ala Asn Xaa Ser Val Phe Leu Lys Lys His Ser Leu Ser Arg Lys Ile	
-15 -10 -5	
TTG GCT GAA AAG TCT TCT TAC AAT TCC GAA TCC CGA CCT TCT ACA GTT	374
Leu Ala Glu Lys Ser Ser Tyr Asn Ser Glu Ser Arg Pro Ser Thr Val	
1 5 10	
GCA GCA TCG GGG AAA GTC CTT GTG CCT GGA ACC CAG AGT GAT AGC TCC	422
Ala Ala Ser Gly Lys Val Leu Val Pro Gly Thr Gln Ser Asp Ser Ser	
15 20 25 30	
TCA TCC TCA ACA GAC CAA TTT GAG TTC ACT GAG ACA TCT CCA GAA AAT	470
Ser Ser Ser Thr Asp Gln Phe Glu Phe Thr Glu Thr Ser Pro Glu Asn	
35 40 45	
TCC CCA GCA TCA ACT GAT GTA GAT AGT TCA ACA ATG GAA CAC GCT AGC	518
Ser Pro Ala Ser Thr Asp Val Asp Ser Ser Thr Met Glu His Ala Ser	
50 55 60	
CAG ATT AAA ACT GAG AAC GAT GAC GTT GAG CCG TCA AGT GAT CTT ACA	566
Gln Ile Lys Thr Glu Asn Asp Asp Val Glu Pro Ser Ser Asp Leu Thr	
65 70 75	
GGA AGT GTT GAA GAG CTG GAT TTT GCT TCA TCA CTA CAA CTA CAA GAA	614
Gly Ser Val Glu Glu Leu Asp Phe Ala Ser Ser Leu Gln Leu Gln Glu	
80 85 90	
GGT GGT AAA CTG GAG GAG TCT AAA ACA TTA AAT ACT TCT GAA GAG AC	662
Gly Gly Lys Leu Glu Glu Ser Lys Thr Leu Asn Thr Ser Glu Glu Thr	
95 100 105 110	
ATT ATT GAT GAA TCT GAT AGG ATC AGA GAG AGG GGC ATC CCT CCA CCT	710
Ile Ile Asp Glu Ser Asp Arg Ile Arg Glu Arg Gly Ile Pro Pro Pro	
115 120 125	
GGA CTT GGT CAG AAG ATT TAT GAA ATA GAC CCC CTT TTG ACA AAC TAT	758
Gly Leu Gly Gln Lys Ile Tyr Glu Ile Asp Pro Leu Leu Thr Asn Tyr	
130 135 140	
CGT CAA CAC CTT GAT TAC AGG TAT TCA CAG TAC AAG AAA CTG AGG GAG	806
Arg Gln His Leu Asp Tyr Arg Tyr Ser Gln Tyr Lys Lys Leu Arg Glu	
145 150 155	

GCA ATT GAC AAG TAT GAG GGT GGT TTG GAA GCT TTT TCT CGT GGT TAT Ala Ile Asp Lys Tyr Glu Gly Gly Leu Glu Ala Phe Ser Arg Gly Tyr 160 165 170	854
GAA AAA ATG GGT TTC ACT CGT AGT GCT ACA GGT ATC ACT TAC CGT GAG Glu Lys Met Gly Phe Thr Arg Ser Ala Thr Gly Ile Thr Tyr Arg Glu 175 180 185 190	902
TGG GCT CCT GGT GCC CAG TCA GCT GCC CTC ATT GGA GAT TTC AAC AAT Trp Ala Pro Gly Ala Gln Ser Ala Ala Leu Ile Gly Asp Phe Asn Asn 195 200 205	950
TGG GAC GCA AAT GCT GAC ATT ATG ACT CGG AAT GAA TTT GGT GTC TGG Trp Asp Ala Asn Ala Asp Ile Met Thr Arg Asn Glu Phe Gly Val Trp 210 215 220	998
GAG ATT TTT CTG CCA AAT AAT GTG GAT GGT TCT OCT GCA ATT CCT CAT Glu Ile Phe Leu Pro Asn Asn Val Asp Gly Ser Pro Ala Ile Pro His 225 230 235	1046
GGG TCC AGA GTG AAG ATA CGT ATG GAC ACT CCA TCA GGT GTT AAG GAT Gly Ser Arg Val Lys Ile Arg Met Asp Thr Pro Ser Gly Val Lys Asp 240 245 250	1094
TCC ATT CCT GCT TGG ATC AAC TAC TCT TTA CAG CTT CCT GAT GAA ATT Ser Ile Pro Ala Trp Ile Asn Tyr Ser Leu Gln Leu Pro Asp Glu Ile 255 260 265 270	1142
CCA TAT AAT GGA ATA TAT TAT GAT CCA CCC GAA GAG GAG AGG TAT ATC Pro Tyr Asn Gly Ile Tyr Tyr Asp Pro Pro Glu Glu Glu Arg Tyr Ile 275 280 285	1190
TTC CAA CAC CCA CGG CCA AAG AAA CCA AAG TCG CTG AGA ATA TAT GAA Phe Gln His Pro Arg Pro Lys Lys Pro Lys Ser Leu Arg Ile Tyr Glu 290 295 300	1238
TCT CAT ATT GGA ATG AGT AGT CCG GAG OCT AAA ATT AAC TCA TAC GTG Ser His Ile Gly Met Ser Ser Pro Glu Pro Lys Ile Asn Ser Tyr Val 305 310 315	1286
AAT TTT AGA GAT GAA GTT CTT CCT CGC ATA AAA AAG CTT GGG TAC AAT Asn Phe Arg Asp Glu Val Leu Pro Arg Ile Lys Lys Leu Gly Tyr Asn 320 325 330	1334
GCG GTG CAA ATT ATG GCT ATT CAA GAG CAT TCT TAT TAT GCT AGT TTT Ala Val Gln Ile Met Ala Ile Gln Glu His Ser Tyr Tyr Ala Ser Phe 335 340 345 350	1382
GGT TAT CAT GTC ACA AAT TTT TTN GCA CCA AGC AGC CGT TTT GGA ACN Gly Tyr His Val Thr Asn Phe Xaa Ala Pro Ser Ser Arg Phe Gly Thr 355 360 365	1430
CCC GAC GAC CTT AAG TCT TTG ATT GAT AAA GCT CAT GAG CTA GGA ATT Pro Asp Asp Leu Lys Ser Leu Ile Asp Lys Ala His Glu Leu Gly Ile 370 375 380	1478
GTT GTT CTC ATG GAC ATT GTT CAC AGC CAT GCA TCA AAT AAT ACT TTA Val Val Leu Met Asp Ile Val His Ser His Ala Ser Asn Asn Thr Leu 385 390 395	1526
GAT GGA CTG AAC ATG TTT GAC GGC ACA GAT AGT TGT TAC TTT CAC TCT Asp Gly Leu Asn Met Phe Asp Gly Thr Asp Ser Cys Tyr Phe His Ser 400 405 410	1574

GGA GCT CGT GGT TAT CAT TGG ATG TGG GAT TCC CGC CTC TTT AAC TAT Gly Ala Arg Gly Tyr His Trp Met Trp Asp Ser Arg Leu Phe Asn Tyr 415 420 425 430	1622
GGA AAC TGG GAG GTA CTT AGG TAT CTT CTC TCA AAT GCG AGA TGG TGG Gly Asn Trp Glu Val Leu Arg Tyr Leu Leu Ser Asn Ala Arg Trp Trp 435 440 445	1670
TTG GAT GAG TTC AAA TTT GAT GGA TTT AGA TTT GAT GGT GTG ACA TCA Leu Asp Glu Phe Lys Phe Asp Gly Phe Arg Phe Asp Gly Val Thr Ser 450 455 460	1718
ATG ATG TAT ACT CAC CAC GGA TTA TCG GTG GGA TTC ACT GGG AAC TAC Met Met Thr His His Gly Leu Ser Val Gly Phe Thr Gly Asn Tyr 465 470 475	1766
GAG GAA TAC TTT GGA CTC GCA ACT GAT GTG GAT GCT GTT GTG TAT CTG Glu Glu Tyr Phe Gly Leu Ala Thr Asp Val Asp Ala Val Val Tyr Leu 480 485 490	1814
ATG CTG GTC AAC GAT CTT ATT CAT GGG CTT TTC CCA GAT GCA ATT ACC Met Leu Val Asn Asp Leu Ile His Gly Leu Phe Pro Asp Ala Ile Thr 495 500 505 510	1862
ATT GGT GAA GAT GTT AGC GGA ATG CCG ACA TTT TNT ATT CCC GTT CAA Ile Gly Glu Asp Val Ser Gly Met Pro Thr Phe Xaa Ile Pro Val Gln 515 520 525	1910
GAT GGG GGT GTT GGC TTT GAC TAT CGG CTG CAT ATG GCA ATT GCT GAT Asp Gly Gly Val Gly Phe Asp Tyr Arg Leu His Met Ala Ile Ala Asp 530 535 540	1958
AAA TGG ATT GAG TTG CTC AAG AAA CGG GAT GAG GAT TGG AGA GTG GGT Lys Trp Ile Glu Leu Leu Lys Lys Arg Asp Glu Asp Trp Arg Val Gly 545 550 555	2006
GAT ATT GTT CAT ACA CTG ACA AAT AGA AGA TGG TCG GAA AAG TGT GTT Asp Ile Val His Thr Leu Thr Asn Arg Arg Trp Ser Glu Lys Cys Val 560 565 570	2054
TCA TAC GCT GAA AGT CAT GAT CAA GCT CTA GTC GGT GAT AAA ACT ATA Ser Tyr Ala Glu Ser His Asp Gln Ala Leu Val Gly Asp Lys Thr Ile 575 580 585 590	2102
GCA TTC TGG CTG ATG GAC AAG GAT ATG TAT GAT TTT ATG GCT CTG GAT Ala Phe Trp Leu Met Asp Lys Asp Met Tyr Asp Phe Met Ala Leu Asp 595 600 605	2150
AGA CCN TCA ACA TCA TTA ATA GAT CGT GGG ATA GCA TTG CAC AAG ATG Arg Pro Ser Thr Ser Leu Ile Asp Arg Gly Ile Ala Leu His Lys Met 610 615 620	2198
ATT AGG CTT GTA ACT ATG GGA TTA GGA GGA GAA GGG TAC CTA AAT TTC Ile Arg Leu Val Thr Met Gly Leu Gly Gly Glu Gly Tyr Leu Asn Phe 625 630 635	2246
ATG GGA AAT GAA TTC GGC CAC CCT GAG TGG ATT GAT TTC CCT AGG GCT Met Gly Asn Glu Phe Gly His Pro Glu Trp Ile Asp Phe Pro Arg Ala 640 645 650	2294
GAA CAA CAC CTC TCT GAT GGC TCA GTA ATT CCC GGA AAC CAA TTC AGT Glu Gln His Leu Ser Asp Gly Ser Val Ile Pro Gly Asn Gln Phe Ser 655 660 665 670	2342



WO 97/20040

PCT/SE96/01558

TAT GAT AAA TGC AGA CGG AGA TTT GAC CTG GGA GAT GCA GAA TAT TTA	2390
Tyr Asp Lys Cys Arg Arg Arg Phe Asp Leu Gly Asp Ala Glu Tyr Leu	
675 680 685	
AGA TAC CGT GGG TTG CAA GAA TTT GAC CGG GCT ATG CAG TAT CTT GAA	2438
Arg Tyr Arg Gly Leu Gln Glu Phe Asp Arg Ala Met Gln Tyr Leu Glu	
690 695 700	
GAT AAA TAT GAG TTT ATG ACT TCA GAA CAC CAG TTC ATA TCA CGA AAG	2486
Asp Lys Tyr Glu Phe Met Thr Ser Glu His Gln Phe Ile Ser Arg Lys	
705 710 715	
GAT GAA GGA GAT AGG ATG ATT GTA TTT GAA AAA GGA AAC CTA GTT TTT	2534
Asp Glu Gly Asp Arg Met Ile Val Phe Glu Lys Gly Asn Leu Val Phe	
720 725 730	
GTC TTT AAT TTT CAC TGG ACA AAA AGC TAT TCA GAC TAT CGC ATA GGC	2582
Val Phe Asn Phe His Trp Thr Lys Ser Tyr Ser Asp Tyr Arg Ile Gly	
735 740 745 750	
TGC CTG AAG CCT GGA AAA TAC AAG GTT GCC TTG GAC TCA GAT GAT CCA	2630
Cys Leu Lys Pro Gly Lys Tyr Lys Val Ala Leu Asp Ser Asp Asp Pro	
755 760 765	
CTT TTT GGT GGC TTC GGG AGA ATT GAT CAT AAT GCC GAA TAT TTC ACC	2678
Leu Phe Gly Gly Phe Gly Arg Ile Asp His Asn Ala Glu Tyr Phe Thr	
770 775 780	
TTT GAA GGA TGG TAT GAT GAT CGT CCT CGT TCA ATT ATG GTG TAT GCA	2721
Phe Glu Gly Trp Tyr Asp Asp Arg Pro Arg Ser Ile Met Val Tyr Ala	
785 790 795	
CCT AGT AGA ACA GCA GTG GTC TAT GCA CTA GTA GAC AAA GAA GAA GAA	2774
Pro Ser Arg Thr Ala Val Val Tyr Ala Leu Val Asp Lys Glu Glu Glu	
800 805 810	
GAA GAA GAA GAA GTA GCA GTA GTA GAA GAA GTA GTA GTA GAA GAA GAA	2822
Glu Glu Glu Glu Val Ala Val Val Glu Glu Val Val Val Glu Glu Glu	
815 820 825 830	
TGA ACGAA CTTGTGATCG CGTTGAAAGA TTTGAAGGCT ACATAGAGCT TCTTGACGTA	2880
***	
TCTGGCAATA TTGCATCACT CTGGCGGAA TTTCATGTGA CAAAAGGTTT GCAATTCTTT	2940
CCACTATTAG TAGTGCAACG ATATACGCAG AGATGAAGTG CTGCACAAAC ATATGTAATA	3000
TCGATGAATT TATGTCGAAT GCTGGGACGG GCTTCAGCAG GTTTTGCTTA GTGAGTTCTG	3060
TAAATGTCA TCTC	3074

SEO ID No. 2

Sequenced molecule: cDNA

Name: beII gene fragment (branching enzyme II) from  
*Solanum tuberosum* (potato)

Length of sequence: 1393 bp

T CTG CCA AAT AAT GTG GAT GGT TCT CCT GCA ATT CCT CAT GGG TCC A	49
Leu Pro Asn Asn Val Asp Gly Ser Pro Ala Ile Pro His Gly Ser Arg	
1 5 10 15	
GTG AAG ATA CGT ATG GAC ACT CCA TCA GGT GTT AAG GAT TCC ATT CCT	97
Val Lys Ile Arg Met Asp Thr Pro Ser Gly Val Lys Asp Ser Ile Pro	
20 25 30	
GCT TGG ATC AAC TAC TCT TTA CAG CTT CCT GAT GAA ATT CCA TAT AAT	145
Ala Trp Ile Asn Tyr Ser Leu Gln Leu Pro Asp Glu Ile Pro Tyr Asn	
35 40 45	
GGA ATA TAT TAT GAT CCA CCC GAA GAG GAG AGG TAT ATC TTC CAA CAC	193
Gly Ile Tyr Tyr Asp Pro Pro Glu Glu Glu Arg Tyr Ile Phe Gln His	
50 55 60	
CCA CGG CCA AAG AAA CCA AAG TCG CTG AGA ATA TAT GAA TCT CAT ATT	241
Pro Arg Pro Lys Lys Pro Lys Ser Leu Arg Ile Tyr Glu Ser His Ile	
65 70 75 80	
GGA ATG AGT AGT CCG GAG CCT AAA ATT AAC TCA TAC GTG AAT TTT AGA	289
Gly Met Ser Ser Pro Glu Pro Lys Ile Asn Ser Tyr Val Asn Phe Arg	
85 90 95	
GAT GAA GTT CTT CCT CGC ATA AAA AAG CTT GGG TAC AAT GCG GTG CAA	337
Asp Glu Val Leu Pro Arg Ile Lys Lys Leu Gly Tyr Asn Ala Val Glr	
100 105 110	
ATT ATG GCT ATT CAA GAG CAT TCT TAT TAT GCT AGT TTT GGT TAT CAT	385
Ile Met Ala Ile Gln Glu His Ser Tyr Tyr Ala Ser Phe Gly Tyr His	
115 120 125	
GTC ACA AAT TTT TTN GCA CCA AGC AGC CGT TTT GGA ACN CCG GAC GAC	433
Val Thr Asn Phe Xaa Ala Pro Ser Ser Arg Phe Gly Thr Pro Asp Asp	
130 135 140	
CTT AAG TCT TTG ATT GAT AAA GCT CAT GAG CTA GGA ATT GTT GTT CTC	481
Leu Lys Ser Leu Ile Asp Lys Ala His Glu Leu Gly Ile Val Val Leu	
145 150 155 160	
ATG GAC ATT GTT CAC AGC CAT GCA TCA AAT AAT ACT TTA GAT GGA CTG	529
Met Asp Ile Val His Ser His Ala Ser Asn Asn Thr Leu Asp Gly Leu	
165 170 175	
AAC ATG TTT GAC GGC ACA GAT AGT TGT TAC TTT CAC TCT GGA GCT CGT	577
Asn Met Phe Asp Gly Thr Asp Ser Cys Tyr Phe His Ser Gly Ala Arg	
180 185 190	
GGT TAT CAT TGG ATG TGG GAT TCC CGC CTC TTT AAC TAT GGA AAC TGG	625
Gly Tyr His Trp Met Trp Asp Ser Arg Leu Phe Asn Tyr Gly Asn Trp	
195 200 205	
GAG GTA CTT AGG TAT CTT CTC TCA AAT GCG AGA TGG TGG TTG GAT GAG	673
Glu Val Leu Arg Tyr Leu Leu Ser Asn Ala Arg Trp Trp Leu Asp Glu	
210 215 220	

TTC AAA TTT GAT GGA TTT AGA TTT GAT GGT GTG ACA TCA ATG ATG TAT	721
Phe Lys Phe Asp Gly Phe Arg Phe Asp Gly Val Thr Ser Met Met Tyr	
225 230 235 240	
ACT CAC CAC GGA TTA TCG GTG GGA TTC ACT GGG AAC TAC GAG GAA TAC	769
Thr His His Gly Leu Ser Val Gly Phe Thr Gly Asn Tyr Glu Glu Tyr	
245 250 255	
TTT GGA CTC GCA ACT GAT GTG GAT GCT GTT GTG TAT CTG ATG CTG GTC	812
Phe Gly Leu Ala Thr Asp Val Asp Ala Val Val Tyr Leu Met Leu Val	
260 265 270	
AAC GAT CTT ATT CAT GGG CTT TTC CCA GAT GCA ATT ACC ATT GGT GAA	865
Asn Asp Leu Ile His Gly Leu Phe Pro Asp Ala Ile Thr Ile Gly Glu	
275 280 285	
GAT GTT AGC GGA ATG CCG ACA TTT TNT ATT CCC GTT CAA GAT GGG GGT	913
Asp Val Ser Gly Met Pro Thr Phe Xaa Ile Pro Val Gln Asp Gly Gly	
290 295 300	
GTT GGC TTT GAC TAT CGG CTG CAT ATG GCA ATT GCT GAT AAA TGG ATT	961
Val Gly Phe Asp Tyr Arg Leu His Met Ala Ile Ala Asp Lys Trp Ile	
305 310 315 320	
GAG TTG CTC AAG AAA CGG GAT GAG GAT TGG AGA GTG GGT GAT ATT GTT	1019
Glu Leu Leu Lys Lys Arg Asp Glu Asp Trp Arg Val Gly Asp Ile Val	
325 330 335	
CAT ACA CTG ACA AAT AGA AGA TGG TCG GAA AAG TGT GTT TCA TAC GCT	1057
His Thr Leu Thr Asn Arg Arg Trp Ser Glu Lys Cys Val Ser Tyr Ala	
340 345 350	
GAA AGT CAT GAT CAA GCT CTA GTC GGT GAT AAA ACT ATA GCA TTC TGG	1105
Glu Ser His Asp Gln Ala Leu Val Gly Asp Lys Thr Ile Ala Phe Trp	
355 360 365	
CTG ATG GAC AAG GAT ATG TAT GAT TTT ATG GCT CTG GAT AGA CCN TCA	1153
Leu Met Asp Lys Asp Met Tyr Asp Phe Met Ala Leu Asp Arg Pro Ser	
370 375 380	
ACA TCA TTA ATA GAT CGT GGG ATA GCA TTG CAC AAG ATG ATT AGG CTT	1201
Thr Ser Leu Ile Asp Arg Gly Ile Ala Leu His Lys Met Ile Arg Leu	
385 390 395 400	
GTA ACT ATG GGA TTA GGA GGA GAA GGG TAC CTA AAT TTC ATG GGA AAT	1249
Val Thr Met Gly Leu Gly Gly Glu Gly Tyr Leu Asn Phe Met Gly Asn	
405 410 415	
GAA TTC GGC CAC CCT GAG TGG ATT GAT TTC CCT AGG GCT GAA CAA CAC	1297
Glu Phe Gly His Pro Glu Trp Ile Asp Phe Pro Arg Ala Glu Gln His	
420 425 430	
CTC TCT GAT GGC TCA GTA ATT CCC GGA AAC CAA TTC AGT TAT GAT AAA	1345
Leu Ser Asp Gly Ser Val Ile Pro Gly Asn Gln Phe Ser Tyr Asp Lys	
435 440 445	
TGC AGA CGG AGA TTT GAC CTG GGA GAT GCA GAA TAT TTA AGA TAC CGT	1393
Cys Arg Arg Arg Phe Asp Leu Gly Asp Ala Glu Tyr Leu Arg Tyr Arg	
450 455 460	

## CLAIMS

1. An amino acid sequence of starch branching enzyme  
5 II (SBE II) comprising the amino acid sequence as shown in  
SEQ ID No. 1.

2. Fragments of the amino acid sequence of starch  
branching enzyme II (SBEII).

3. A fragment according to claim 2, having the amino  
10 acid sequence as shown in SEQ ID No. 2.

4. An isolated DNA sequence encoding starch branching  
enzyme II (SBE II) of potato comprising the nucleotide  
sequence as shown in SEQ ID No. 1 variants thereof  
resulting from the degeneracy of the genetic code.

15 5. Fragments of the isolated DNA sequence encoding  
starch branching enzyme II (SBEII) of potato.

6. A fragment according to claim 5, comprising the  
nucleotide sequence as shown in SEQ ID No. 2.

7. A vector comprising the whole or a functionally  
20 active part of the isolated DNA sequence claimed in any  
one of claims 4-6 and regulatory elements active in  
potato.

8. A vector according to claim 7, wherein the DNA  
sequence is in the antisense (reversed) orientation in  
25 relation to a promoter immediately upstream from the DNA  
sequence.

9. A process for the production of transgenic  
potatoes with either an increased or a decreased degree of  
branching of amylopectin starch, characterized  
30 in that it comprises the following steps:  
a) transfer and incorporation of a vector according to  
claim 7 into the genome of a potato cell, and  
b) regeneration of intact, whole plants from the  
transformed cells.

35 10. A process for the production of transgenic  
potatoes with a reduced degree of branching of amylopectin

starch, characterized in that it comprises the following steps:

- a) transfer and incorporation of a vector according to claim 8 into the genome of a potato cell, and
- 5 b) regeneration of intact, whole plants from the transformed cells.

11. A process according to claim 10, wherein the vector also comprises an antisense construct of starch branching enzyme I (SBE I).

- 10 12. A process according to claims 10 or 11, wherein the vector also comprises an antisense construct of potato granule bound starch synthase II.

- 13. A process according to one or more of claims 10-12, wherein the vector also comprises an antisense  
15 construct of potato soluble starch synthases II and III.

14. A process according to one or more of claims 10-13, wherein the vector also comprises an antisense construct of potato starch disproportionating enzyme (D-enzyme).

- 20 15. A process according to one or more of claims 10-14, wherein the vector also comprises an antisense construct of potato starch debranching enzyme.

16. A transgenic potato obtainable by the process according to any one of claims 9-15.

- 25 17. Use of transgenic potatoes according to claim 16 for the production of starch.

1/2

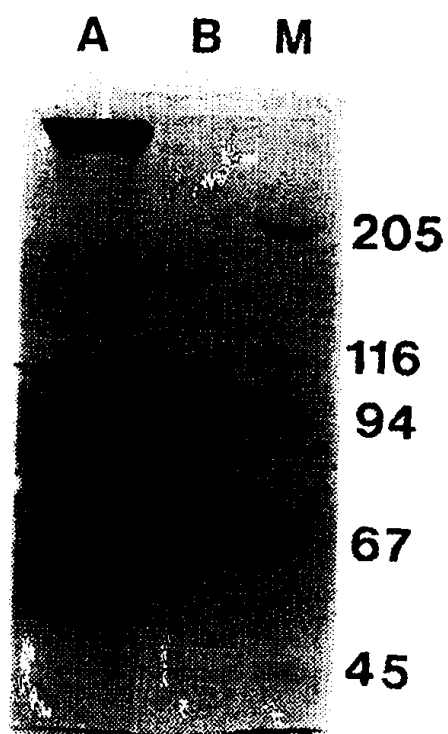


FIG 1

**SUBSTITUTE SHEET**

## FIG. 2

Peptide 1. EFGVWEIFLPN

Peptide 2. HGLQEFDRA

Peptide 3. ENDGIAAKADE

Peptide 4. YEIDPEI/LTN

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 96/01558

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/10, C12N 15/82, A01H 5/06

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS, EMBL/GENBANK/DBJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9504826 A1 (INSTITUT FÜR GENBIOLOGISCHE FORSCHUNG BERLIN GMBH), 16 February 1995 (16.02.95), see abstract and claim 23 --	1-17
X	WO 9214827 A1 (INSTITUT FÜR GENBIOLOGISCHE FORSCHUNG BERLIN GMBH), 3 Sept 1992 (03.09.92), see page 5, line 1-7 and examples --	1-17
A	SE 467160 B (AMYLOGENE HANDELSBOLAG), 1 June 1992 (01.06.92) -- -----	1-17

☐ Further documents are listed in the continuation of Box C.
 ☒ See patent family annex.

- \* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search  27 February 1997	Date of mailing of the international search report 01-03-1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer  Yvonne Siösteen Telephone No. -46 8 782 25 00



**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

PCT/SE 96/01558

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9504826	16/02/95	AU-A- 7535294	28/02/95
		EP-A- 0713531	29/05/96
		CA-A- 2169174	16/02/95
		DE-A- 4327165	16/02/95
		HU-A- 73740	30/09/96
		HU-D- 9600285	00/00/00
		IL-D- 110583	00/00/00
WO-A1- 9214827	03/09/92	AU-B- 663072	28/09/95
		AU-A- 1226592	15/09/92
		CA-A- 2104123	14/08/92
		DE-A- 4104782	20/08/92
		EP-A- 0571427	01/12/93
		HU-A- 65740	28/07/94
SE-B- 467160	01/06/92	AU-A- 9109791	22/07/92
		EP-A- 0563201	06/10/93
		PL-B- 169859	30/09/96
		SE-A- 9004095	01/06/92
		WO-A- 9211375	09/07/92